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GRANT NUMBER DAMD17-94-J-4335

TITLE: The Role of Integrin Signaling in Breast Cancer

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REPORT DATE: October 1997

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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OMB No. 0704-0188

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1997	3. REPORT TYPE AND DATES COVERED Final (16 Sep 94 - 15 Sep 97)	
4. TITLE AND SUBTITLE The Role of Integrin Signaling in Breast Cancer			5. FUNDING NUMBERS DAMD17-94-J-4335	
6. AUTHOR(S) Leslie M. Shaw, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Beth Israel Deaconess Medical Center, Inc. Boston, Massachusetts 02215			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, Oct 97). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) During this fellowship award, I made considerable progress in understanding the contribution of the $\alpha 6$ integrin receptors to breast carcinoma progression. I developed a dominant negative strategy to 'knock-out' the expression of the $\alpha 6 \beta 1$ integrin in a highly metastatic breast carcinoma cell line (MDA-MB-435). After depletion of $\alpha 6 \beta 1$ surface expression, these cells are deficient in their growth in the mammary fat pad of athymic mice and can no longer survive as metastases in the lungs and liver. These findings suggest that the $\alpha 6 \beta 1$ integrin plays an important role in regulating the growth and survival of breast carcinoma. I also established MDA-MB-435 transfectants that express the $\alpha 6 \beta 4$ integrin receptor. These cells have a marked increase in their ability to invade through basement membrane matrices <i>in vitro</i> . I determined that the mechanism by which this integrin promotes invasion involves a preferential and localized targeting of phosphoinositide-3 OH kinase (PI 3-K) activity. The small GTP-binding protein Rac is downstream of PI 3-K in the cells examined and it is involved in invasion. Collectively, these findings provide a mechanism for the involvement of $\alpha 6 \beta 4$ in promoting carcinoma invasion and invoke a novel function for PI 3-K signaling.				
14. SUBJECT TERMS Breast Cancer Integrins, Laminin, Phosphoinositide-3 OH Kinase, Apoptosis, Invasion, Metastasis			15. NUMBER OF PAGES 17	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited	

FOREWORD

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Leslie M. Shaw 10/14/97
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Introduction

A major advance in cancer research made during the last several years has been the realization that integrin adhesion receptors play a major role in regulating tumor behavior. Specific tumor-associated functions that are regulated by integrins include adhesion and migration, differentiation, growth and apoptosis, and angiogenesis (1-5). This array of diverse but important functions reflects the fact that integrins are critical for the function and maintenance of the normal epithelial progenitors of all carcinomas. The importance of integrin-mediated interactions in normal epithelial biology has been particularly well-demonstrated for the mammary epithelium (6). Such studies have identified the laminins, a family of extracellular matrix proteins, as key players in determining the function of both normal and transformed mammary epithelia. For these reasons, integrin laminin receptors are prime candidates for investigating the role of cell adhesion events in breast carcinoma (7).

Although several integrin heterodimers can function as receptors for members of the laminin family, the $\alpha 6$ integrins, $\alpha 6\beta 1$ and $\alpha 6\beta 4$, are the predominant laminin receptors in many cell types (7). The possibility that the $\alpha 6$ integrins play an important role in breast carcinoma progression is supported by a study by Imhof and colleagues (8) which examined the expression of $\alpha 6$ in invasive breast carcinomas of 119 women and correlated levels of expression with patient survival. The results demonstrated that high expression levels of the $\alpha 6$ subunit correlated with reduced patient survival time. In fact, $\alpha 6$ integrin expression was superior in predicting reduced survival than other known factors alone including estrogen receptor status. These data implicate a key role for the $\alpha 6$ integrins in deadly breast carcinomas. Data to support the involvement of the $\alpha 6$ integrins in the progression of other carcinomas has also been reported. Expression of the $\alpha 6$ integrins in prostate carcinoma cell lines correlates directly with the invasive potential of these cells in nude mice (9). In addition, expression of the $\alpha 6\beta 4$ integrin has been reported to correlate with the progression of squamous, gastric, and colon carcinomas (10-12). Taken together, these data, in conjunction with the Imhof data, provide a compelling case for the involvement of $\alpha 6$ integrins in carcinoma progression. However, these studies only emphasize the potential importance of the $\alpha 6$ integrins in breast cancer, but they do not provide mechanistic information. Until this is established, the full potential of $\alpha 6$ for diagnosis, or as a target for therapeutic development, will not be known.

The ability of integrin receptors to influence cellular processes such as growth, differentiation, cell motility, and tumor progression is the result of both their adhesive and signaling capabilities (1,13). Many of the signaling pathways that have been characterized for growth factor receptors can be stimulated by integrin receptor engagement. In fact, these two receptor systems may act in concert to elicit the specific signals that are required for proper cell function (14). There is still much to be learned about how integrin receptors control signaling pathways but it is clear that alterations in integrin expression and function during transformation are likely to have multiple consequences on tumor progression. The purpose of the work supported by this fellowship was to elucidate specific functions of breast carcinoma cells that are influenced by the $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins and to elucidate the signaling pathways that control these functions. The work performed under this award has made significant progress toward attaining this goal.

Body

The $\alpha 6 \beta 1$ integrin is required for the growth and survival of MDA-MB-435 breast carcinoma cells *in vivo*. To examine the contribution of the $\alpha 6 \beta 1$ integrin to breast carcinoma progression, I developed a dominant-negative technique for 'knocking-out' the expression of $\alpha 6 \beta 1$ in MDA-MB-435 cells, a human breast carcinoma cell line that is highly metastatic in athymic mice. A mutant $\beta 4$ subunit that lacked the entire cytoplasmic domain was expressed at high levels in this cell line. $\beta 4$ dimerizes only with $\alpha 6$, and not other integrin α subunits, and therefore it selectively depleted $\alpha 6 \beta 1$ surface expression by forming the $\alpha 6 \beta 4$ - Δ CYT heterodimer. I found that elimination of $\alpha 6 \beta 1$ expression inhibited the ability of these cells to mediate specific *in vitro* functions associated with metastatic spread such as laminin adhesion and migration (Described in previous Annual Report; Shaw, L.M., C. Chao, U.M. Wewer, and A.M. Mercurio. 1996. *Function of the integrin $\alpha 6 \beta 1$ in metastatic breast carcinoma cells assessed by expression of a dominant negative receptor. Cancer Res., 56:959-963.*). Subclones of these transfectants were isolated by FACS sorting. Mock (transfected with the vector alone) and $\beta 4$ - Δ CYT transfectant subclones were chosen that expressed similar levels of all integrin subunits. These subclones also adhered to other matrix proteins such as fibronectin, vitronectin, and collagen I to the same extent.

In collaboration with Ulla Wewer and Reidar Albrechtsen in Copenhagen, Denmark, I examined the *in vivo* behavior of MDA-MB-435 breast carcinoma cells that lack $\alpha 6 \beta 1$ expression. Mock and $\alpha 6 \beta 4$ - Δ CYT transfectant subclones were injected into the mammary fat pad of female athymic mice. After several weeks, the mice were sacrificed and the extent of tumor growth, invasion, and metastasis was analyzed. The tumors formed by the $\alpha 6 \beta 4$ - Δ CYT transfectant subclones were significantly smaller than those formed by the mock transfectants and metastatic foci were not present in the lungs of the $\beta 4$ - Δ CYT transfectant mice. However, small foci of apoptotic cells were observed in the lungs of the mice injected with the $\alpha 6 \beta 4$ - Δ CYT transfectant subclones and a high level of apoptosis was observed in the primary tumors (Described in previous Annual Report; Wewer, U.M., L.M. Shaw, R. Albrechtsen, and A.M. Mercurio. 1997. *The integrin $\alpha 6 \beta 1$ promotes the survival of metastatic human breast carcinoma cells in mice. Amer. J. Path. (in press)*). These data suggest that the $\beta 4$ - Δ CYT transfectants were able to metastasize to the lungs but were not capable of surviving in this environment. Taken together, these data indicate that the $\alpha 6 \beta 1$ integrin functions in the growth and survival of breast carcinomas.

The $\alpha 6 \beta 1$ integrin can activate MAPK To begin to understand how the $\alpha 6 \beta 1$ integrin functions in the growth and survival of breast carcinomas, I examined the ability of this integrin to activate signaling pathways that regulate these functions. The Mitogen activated protein kinases (MAPK) have been shown to be important for growth control because they regulate the expression of immediate early genes required for cell cycle progression (15). It was recently reported that only a subset of integrin receptors can activate MAPK and that the $\alpha 6 \beta 1$ integrin was not included in this group (16). To determine if the ability of $\alpha 6 \beta 1$ to activate MAPK was cell type dependent, I assayed MAPK activity after ligation of $\alpha 6 \beta 1$ in the MDA-MB-435 cells. As shown in Fig. 1, ligation of $\alpha 6 \beta 1$ by clustering with antibodies and attachment to laminin induced MAPK activity in these cells. Interestingly, ligation of the $\alpha 6 \beta 4$ - Δ CYT receptor did not induce activation of MAPK confirming that this receptor is non-functional for signaling. This inability of the $\alpha 6 \beta 4$ - Δ CYT receptor to activate MAPK could explain the decrease in the growth of these tumors.

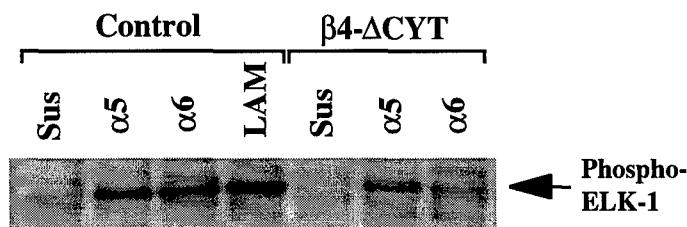
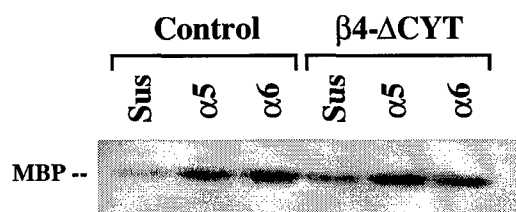


Figure 1: Activation of MAPK by the $\alpha 6 \beta 1$ integrin. MDA-MB-435 transfectants were maintained in suspension or incubated with $\alpha 6$ - or $\alpha 5$ -specific antibodies and allowed to adhere to anti-mouse IgG coated plates or laminin-1 coated plates for 30 minutes. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with an anti-phosphoMAPK antibody and then Protein A sepharose. After washing, the beads were resuspended in kinase buffer containing the Elk-1 substrate. The reactions were resolved by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and immunoblotted using an antibody that recognizes the phosphorylated Elk-1.

The $\alpha 6 \beta 1$ integrin can activate S6 kinase and Akt kinase It has recently been determined that the activity of the Akt serine/threonine kinase is required for survival of several different cell types (17,18). Given the increased apoptosis that was observed in the primary tumors formed by the MDA-MB-435 cells lacking $\alpha 6 \beta 1$, we chose to examine if $\alpha 6 \beta 1$ could activate Akt. Initially, we examined the activation of the p70 S6 kinase because this kinase is known to be downstream of Akt activity (19). As shown in Fig. 2A, ligation of both the $\alpha 6 \beta 1$ and $\alpha 5 \beta 1$ integrins activated S6 kinase. We next assayed for Akt kinase activity after ligation of $\alpha 6 \beta 1$. As shown in Fig. 2B, Akt was activated after 20 minutes of clustering the receptor with $\alpha 6$ specific antibodies. Importantly, neither kinase was activated upon clustering the $\alpha 6 \beta 4$ - Δ CYT receptor, as was observed for activation of MAPK.

A



B

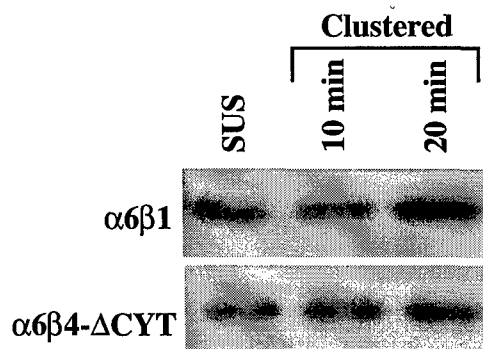


Figure 2: The $\alpha 6 \beta 1$ integrin activates p70 S6 kinase and Akt kinase. MDA-MB-435 transfectants were maintained in suspension or incubated with $\alpha 6$ - or $\alpha 5$ -specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 minutes. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with an anti-p70 S6 kinase (A) or anti-Akt (B) antibody and then Protein A sepharose. After washing, the beads

were resuspended in kinase buffer containing MBP (A) or histone H2B (B). The reactions were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose.

The $\alpha 6 \beta 1$ integrin cooperates with growth factors for activation of signaling pathways The ability of the $\alpha 6 \beta 1$ integrin to activate MAPK, p70-S6 Kinase, and Akt support the hypothesis that this integrin can contribute to the growth and survival of breast carcinoma cells. However, other integrins, such as $\alpha 5 \beta 1$, were also capable of activating these important pathways. Therefore, the question arises as to why $\alpha 6 \beta 1$ is necessary for breast carcinoma growth and survival *in vivo* when other integrin receptors can also activate these pathways. It has been demonstrated previously that integrins can cooperate with growth factor receptors to enhance the activation of downstream signaling pathways (14). Therefore, we examined the possibility that $\alpha 6 \beta 1$ cooperates preferentially with a growth factor signaling pathway to enhance the signals required for growth and survival. As shown in Fig. 3, Insulin-like growth factor 1 (IGF-1) stimulates very little Akt activity when added to cells that are maintained in suspension. However, a marked increase in activity is observed if integrin receptors are ligated when the IGF-1 is added. Importantly, Akt activity was consistently higher when $\alpha 6 \beta 1$ was ligated in the presence of IGF-1 than when other integrin receptors were ligated (Fig. 3). This cooperative effect was also observed for p70 S6 kinase. These data support the possibility that $\alpha 6 \beta 1$ is important for breast carcinoma survival because this integrin cooperates better with growth factors to activate the signaling pathways that prevent apoptosis, such as those that regulate Akt. Future experiments will continue to examine this cooperation between $\alpha 6 \beta 1$ and growth factors and their role in promoting breast carcinoma growth and survival.

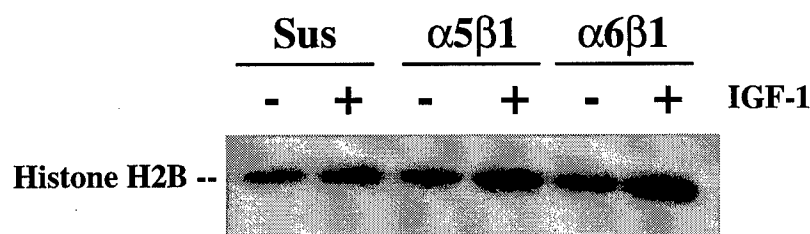


Figure 3: MDA-MB-435 transfectants were maintained in suspension or incubated with $\alpha 6$ - or $\alpha 5$ -specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 minutes. IGF-1 was added to some of the plates for the final 10 minutes. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with an anti-Akt antibody and then Protein A sepharose. After washing, the beads were resuspended in kinase buffer containing histone H2B. The reactions were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose.

Expression of the $\alpha 6 \beta 4$ integrin increases the invasiveness of MDA-MB-435 cells Many studies have implicated the $\alpha 6 \beta 4$ integrin in carcinoma progression, however, the mechanism is unknown (9-12)). The MDA-MB-435 cells used in this study do not express the $\alpha 6 \beta 4$ integrin although they express the $\alpha 6 \beta 1$ integrin. To examine the contribution of $\alpha 6 \beta 4$ to breast carcinoma progression, MDA-MB-435 cells were transfected with the full length $\beta 4$ subunit and stable subclones of these cells that express the $\alpha 6 \beta 4$ integrin were isolated (Described in previous Annual Report). These transfectants were examined for their ability to invade Matrigel in a standard chemoinvasion assay. The rate of invasion of the $\beta 4$ transfectants was approximately 3-4 fold greater than that of the mock transfectants in a 4 hour assay. The $\beta 4$ - Δ CYT transfectants

invaded at a slightly slower rate than that of the mock transfectants (Fig. 4A) indicating that the $\beta 4$ cytoplasmic domain is essential for stimulating invasion. To examine the contribution of integrin receptors to the invasion of MDA-MB-435 cells, Matrigel chemoinvasion assays were performed in the presence of integrin subunit specific antibodies. A $\beta 1$ -specific antibody (mAb 13) inhibited invasion of the mock and $\beta 4$ -transfectants (Fig. 4B). An $\alpha 6$ -specific mAb (2B7) inhibited invasion of the mock transfectants by approximately 60% (Fig. 4B), in agreement with our previous result that these cells use $\alpha 6\beta 1$ as a major laminin receptor. However, the same antibody increased the rate of invasion of the $\beta 4$ transfectants by approximately 30% (Fig. 4B). The stimulation of invasion observed for the $\alpha 6$ antibody in the MDA-MB-435/ $\beta 4$ transfectants suggests that $\alpha 6\beta 4$ is not required for the adhesive functions involved in invasion, but rather acts as a signaling receptor whose function can be enhanced by antibody binding.

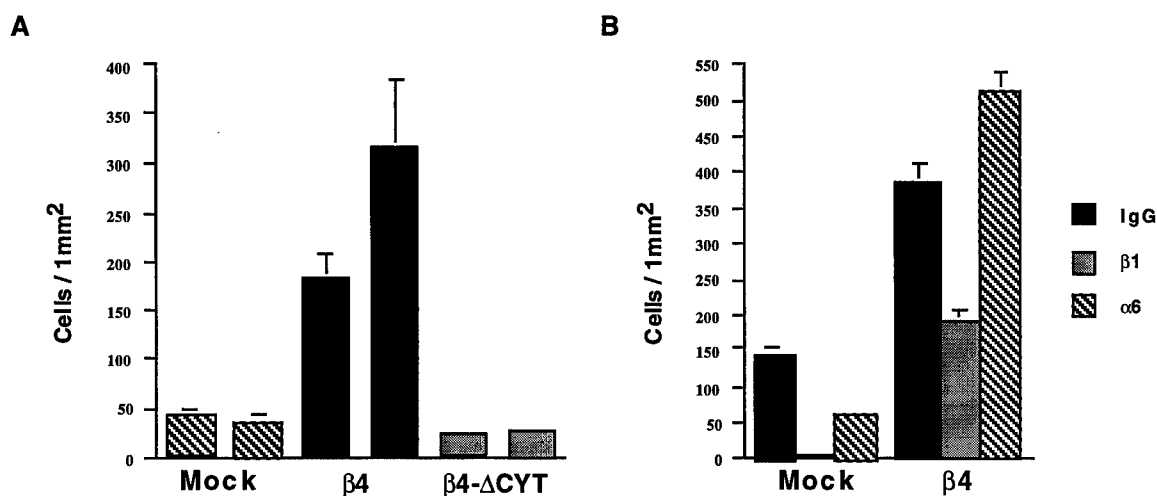


Figure 4: Invasion of the MDA-MB-435 transfectants. A) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel. Matrigel was diluted in cold distilled water, added to the upper well of Transwell chambers, and dried in a sterile hood. The Matrigel was reconstituted with medium and the transfectants were added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers. B) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel in the presence of monoclonal antibodies specific for the indicated integrin subunits. Cells were preincubated for 30 minutes in the presence of antibodies before addition to the Matrigel-coated wells. After 4 hrs, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated. Mock, MDA-MB-435 cells transfected with vector alone; $\beta 4$, MDA-MB-435 cells transfected with the full length $\beta 4$ subunit; $\beta 4$ - Δ CYT, MDA-MB-435 cells transfected with the $\beta 4$ subunit lacking the cytoplasmic domain; IgG; non-specific antibody; $\beta 1$, mAb 13; $\alpha 6$, 2B7.

Invasion of MDA-MB-435 cells is dependent on PI 3-K. As a prelude to identifying the signaling mechanism by which the $\alpha 6\beta 4$ integrin stimulates invasion, we first assessed the effects of the MAPK kinase inhibitor PD98059 on MDA-MB-435 invasion (20). We selected this pathway because it has been reported that MAPK contributes to cell motility by phosphorylating myosin light chain kinase (). As shown in Fig. 5, pre-treatment of these cells with PD98059 (25 μ M) resulted in only a modest inhibition (20%) of invasion. We next targeted PI 3-K because of its central involvement in multiple signaling pathways (21). The PI 3-K inhibitor wortmannin

inhibited invasion of both the mock and $\beta 4$ transfectants by 60-80% (22; Fig. 5). These results suggest that PI 3-K, but not MAPK, is necessary for the invasion of the MDA-MB-435 cells.

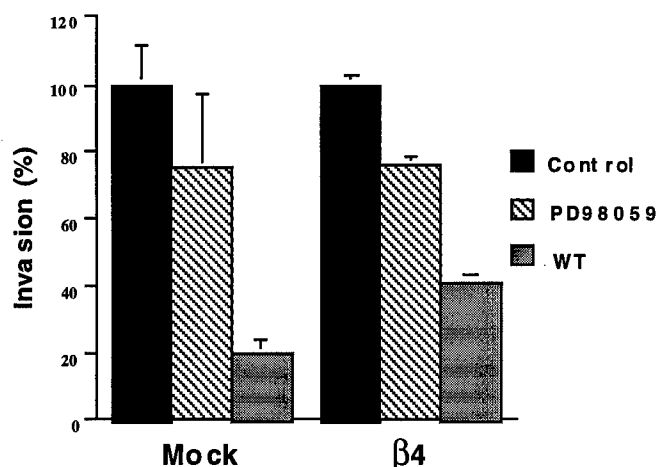


Figure 5: Analysis of MAPK and PI 3-K involvement in MDA-MB-435 invasion. MDA-MB-435 transfectants were assayed for their ability to invade Matrigel in the presence of the MEK inhibitor PD98059 (25 μ M) or the PI 3-K inhibitor wortmannin (WT;100 μ M). Cells were preincubated for 10 minutes in the presence of the inhibitors before addition to the Matrigel-coated wells. After 4 hrs, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated. The data shown are the mean values (\pm SD) of one experiment done in duplicate. Mock, MDA-MB-435 cells transfected with vector alone; $\beta 4$, MDA-MB-435 cells transfected with the full length $\beta 4$ subunit.

Activation of PI 3-K by the $\alpha 6\beta 4$ integrin To determine if the $\alpha 6\beta 4$ integrin can stimulate PI 3-K activity, *in vitro* kinase assays were performed using the mock, $\beta 4$, and $\beta 4$ - Δ CYT transfectants of MDA-MB-435 cells. After ligation of the $\alpha 6$ integrins with 2B7, extracts were immunoprecipitated with a phosphotyrosine-specific antibody to capture the activated population of PI 3-K and these immunoprecipitates were assayed for their ability to phosphorylate crude brain phosphoinositides. As shown in Fig. 6A, an increase in PI 3-K activity, indicated by the appearance of PtdIns-3,4,5- P_3 , was observed upon clustering the $\alpha 6\beta 1$ integrin in the mock transfectants and the $\alpha 6\beta 4$ integrin in the $\beta 4$ transfectants. More importantly, PI 3-K activity stimulated by clustering the $\alpha 6\beta 4$ integrin was markedly greater than that observed after clustering the $\alpha 6\beta 1$ receptor. This enhanced stimulation of PI 3-K was also seen using a $\beta 4$ -specific mAb to ligate the $\alpha 6\beta 4$ integrin in the $\beta 4$ transfectants (Fig. 6A). PI 3-K activity was also higher in the $\beta 4$ transfectants than in the mock transfectants after adhesion to laminin-1 (Fig. 6A). This observation suggests that even though $\alpha 6\beta 4$ is not used as the major adhesion receptor in these cells, interactions with laminin through this receptor can stimulate PI 3-K activity. PI 3-K activity was not increased upon ligation of the $\alpha 6\beta 4$ - Δ CYT receptor and little activity was evident when the transfectants were maintained in suspension (Fig. 6).

Our data suggested that the ability of the $\alpha 6\beta 4$ integrin to activate PI 3-K may be quantitatively greater than that of $\beta 1$ integrins in MDA-MB-435 cells. This possibility was examined by comparing PI 3-K activation in the $\beta 4$ transfectants in response to antibody-ligation of either $\beta 1$ integrins or the $\alpha 6\beta 4$ -integrin. As shown in Fig. 6B, ligation of the $\alpha 6\beta 4$ integrin with $\beta 4$ -specific antibodies stimulated PI 3-K activity approximately two fold greater than $\beta 1$ integrin ligation demonstrating that PI 3-K is activated preferentially by the $\alpha 6\beta 4$ integrin. The differences between the abilities of the $\alpha 6\beta 4$ and $\beta 1$ integrins to activate PI 3-K are most likely even greater

than what was observed given the two-three fold higher level of expression of $\beta 1$ than $\beta 4$ on the cell surface.

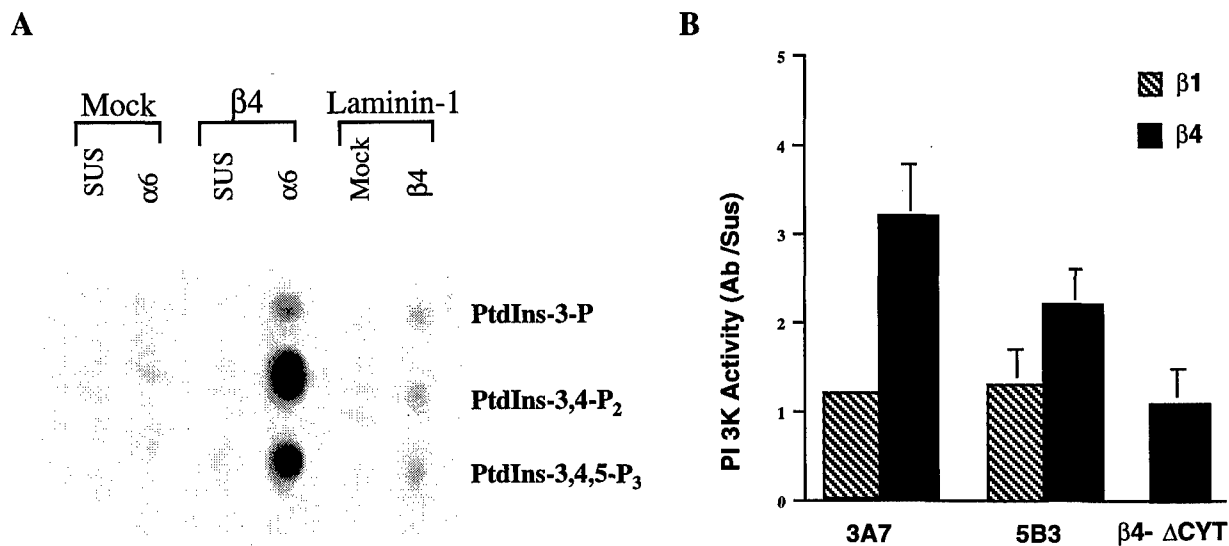


Figure 6: Analysis of PI 3-K activity in the MDA-MB-435 transfectants. MDA-MB-435 transfectants were maintained in suspension or incubated with $\alpha 6$ -, $\beta 1$ -, or $\beta 4$ -specific antibodies and allowed to adhere to anti-mouse IgG coated plates or laminin-1 coated plates for 30 minutes. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-phosphotyrosine mAb 4G10 and Protein A sepharose for 3hrs. After washing, the beads were resuspended in kinase buffer and incubated for 10 minutes at room temperature. The phosphorylated lipids were resolved by thin layer chromatography. A) The D3-phosphoinositides are indicated by arrows. B) The amount of radiolabeled PtdIns-3,4,5- P_3 was determined for each condition by densitometry. The integrin activated levels of PtdIns-3,4,5- P_3 were compared to the level observed for the cells that were maintained in suspension. The value from this ratio was determined to be the relative PI 3-K activity stimulated by each integrin subunit. Mock, MDA-MB-435 cells transfected with vector alone; $\beta 4$, MDA-MB-435 cells transfected with the full length $\beta 4$ subunit; SUS, cells maintained in suspension; $\alpha 6$, cells clustered with the $\alpha 6$ -specific antibody.

Constitutively active PI-3K stimulates invasion of MDA-MB-435 cells The hypothesis that the $\alpha 6\beta 4$ integrin promotes invasion of MDA-MB-435 cells by enhancing the activity of PI 3-K implies that: 1) expression of a constitutively active form of PI 3-K in the parental cells should increase their invasion in the absence of $\alpha 6\beta 4$ expression and 2) expression of a dominant negative PI 3-K subunit in the $\beta 4$ transfectants should decrease their invasion. To validate these predictions, a constitutively active, membrane-targeted PI 3-K (Myr-p110-Myc) was expressed transiently in MDA-MB-435 cells and the ability of these cells to invade Matrigel was compared to cells transfected with an empty vector. As shown in Fig. 7A, constitutively active PI 3-K increased invasion two-fold and this invasion was inhibited by wortmannin. Overexpression of the wild type p85 regulatory subunit has been demonstrated to function in a dominant negative manner and inhibit endogenous PI 3-K activation (23). Transient expression of a myc-tagged p85

subunit inhibited the invasion of the MDA-MB-435/ β 4 transfectants by 50% (Fig. 7B). The data obtained with the wild-type p85 subunit substantiate the wortmannin data shown in Fig. 5.

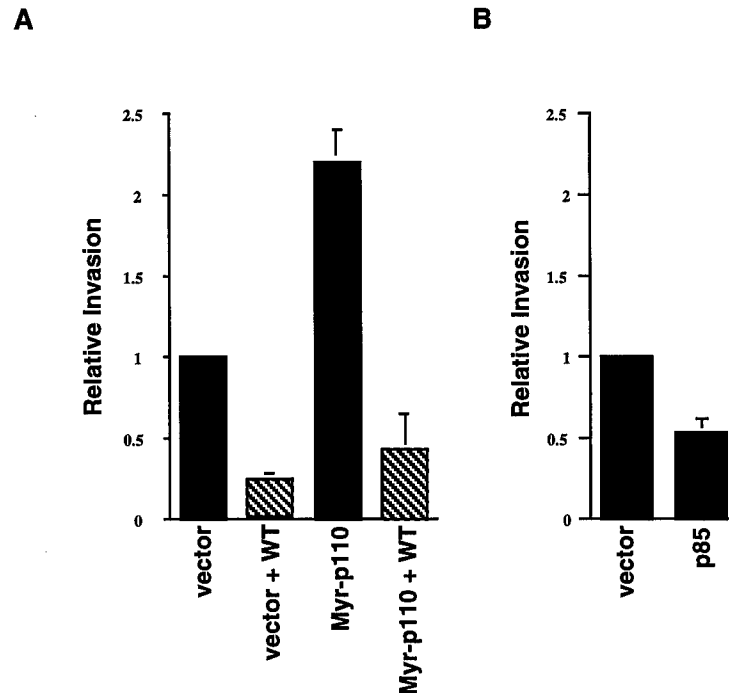


Figure 7: Analysis of PI 3-K involvement in invasion of MDA-MB-435 cells by transient transfections. A) MDA-MB-435 cells were transiently transfected with a Myc-tagged constitutively active form of the PI 3-K p110 catalytic subunit, Myr-p110, and assayed for their ability to invade Matrigel in the absence or presence of wortmannin (WT; 100nM). B) MDA-MB-435/b4 transfectants were transiently transfected with a wild-type PI 3-K p85 regulatory subunit, p85, and assayed for their ability to invade Matrigel. After 5 hrs, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed and stained. Invasion was quantitated by counting the cells that stained positively for β -galactosidase expression. Relative invasion was determined by comparing the amount of invasion obtained for the experimental transfections to that observed for the cells transfected with the vector alone, which was given the value of 1.

The Akt/PKB kinase, a downstream effector of PI-3K, is not required for invasion The Akt/PKB serine/threonine kinase (Akt) is activated downstream of PI 3-K and, for this reason, could play an important role in invasion (24). This possibility was supported by our finding that ligation of the α 6 β 4 integrin in the MDA-MB-435 transfectants can activate Akt. Based on these observations, we examined the ability of MDA-MB-435 cells that expressed a constitutively active form of Akt (Myr-Akt) to invade Matrigel. Surprisingly, this constitutively active form of Akt actually decreased the rate of invasion in comparison to the control cells even though it was expressed at relatively high levels (Fig. 8A). Most likely, the exogenously expressed Akt sequestered a significant fraction of D3 phosphoinositides and precluded the use of these lipids in those signaling pathways downstream of PI 3-K that are required for invasion. Based on these results, we conclude that Akt is not required for MDA-MB-435 invasion.

The small G-protein Rac is required for MDA-MB-435 invasion The small G-protein Rac is a downstream effector of PI 3-K that is involved in the actin rearrangements that result in the formation of membrane ruffles and lamellae (25). The ability of cells to form lamellae is strongly correlated with their motility and therefore influences their invasive potential. To examine the role of Rac in PI 3-K dependent invasion, a dominant negative mutant of Rac, N17Rac, was transiently expressed along with the Myr-p110-Myc construct in the MDA-MB-435 cells. As shown in Fig. 8A, co-expression of the N17Rac inhibited the increased invasion that was observed when the constitutively active p110 subunit of PI 3-K was expressed alone. In addition to this experiment, the N17Rac construct was expressed transiently in the MDA-MB-435/ β 4 transfectants and a 50% reduction in invasion was observed (Fig. 8B). Taken together, these results demonstrate that Rac is an essential downstream mediator of the α 6 β 4/PI 3-K signaling pathway involved in invasion. Expression of a constitutively active mutant of Rac, V12Rac, did not significantly increase the invasion of the MDA-MB-435 cells suggesting that other PI-3K downstream effectors, in addition to Rac, are important for invasion in these cells. (data not shown).

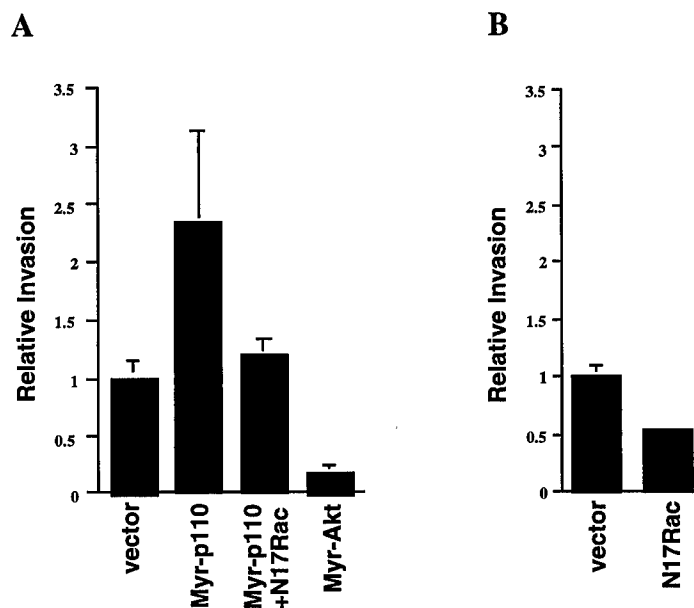


Figure 8: Analysis of Rac and Akt involvement in PI 3-K-dependent invasion of MDA-MB-435 cells. A) MDA-MB-435 cells were transiently transfected with either the vector alone, Myr-p110, Myr-p110 and a dominant negative mutant of Rac, GST-N17Rac, or a constitutively active form of Akt, Myr-Akt, and assayed for their ability to invade Matrigel. B) MDA-MB-435/ β 4 transfectants were transiently transfected with vector alone or N17Rac and assayed for their ability to invade Matrigel. After 5 hrs, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed and stained. Invasion was quantitated by counting the cells that stained positive for β -galactosidase expression. Relative invasion was determined by comparing the amount of invasion obtained for the experimental transfections to that observed for the cells transfected with the vector alone, which was given the value of 1.

Conclusions

In order to take full advantage of the $\alpha 6$ subunit as a marker for predicting the prognosis of breast cancer, it is necessary to understand mechanistically how the $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins promote aggressive tumor behavior. Until this is established, the full potential of $\alpha 6$ for diagnosis, or as a target for therapeutic development, will not be known. The goal of this postdoctoral fellowship was to examine the role of the $\alpha 6$ integrins in breast carcinoma progression. Extensive progress has been made toward developing an understanding of the importance of these receptors in breast carcinoma biology. Although the original aims of this proposal were to compare the cytoplasmic domain variants of the $\alpha 6$ subunit, early studies directed the research in different, exciting directions. The work from this award has demonstrated that the $\alpha 6$ integrins have specific roles in breast carcinoma progression that involve distinct signaling functions of $\alpha 6\beta 1$ and $\alpha 6\beta 4$.

Elimination of $\alpha 6\beta 1$ expression from a highly metastatic breast carcinoma cell line inhibited the ability of these cells to metastasize in athymic mice. However, this was not due to an inability to invade as was predicted from *in vitro* experiments that showed a decreased ability to adhere and migrate on laminin substrates. Rather, the decreased metastasis appears to be due to the inability of these cells to survive in distant organs. These cells are also inhibited in their primary tumor growth because of a decreased proliferative rate and an increased apoptotic rate. Therefore, the conclusion can be drawn that the $\alpha 6\beta 1$ integrin contributes to breast carcinoma progression by controlling signaling pathways that are involved in cell cycle progression and/or apoptotic regulation. The work in the final year of this fellowship has focused on identifying specific signaling pathways that are activated by the $\alpha 6\beta 1$ integrin that would contribute to these functions. The data indicate that the $\alpha 6\beta 1$ integrin may cooperate with growth factor receptors to enhance growth and survival signals such as the activation of Akt and p70 S6 kinase.

The $\alpha 6\beta 4$ integrin regulates signaling pathways that influence carcinoma invasion. Expression of the $\beta 4$ integrin subunit in a highly metastatic breast carcinoma cell line markedly increased the *in vitro* invasive potential of these cells. The mechanism by which this integrin promotes invasion involves a preferential and localized targeting of phosphoinositide-3 OH kinase (PI 3-K) activity. Exogenous expression of $\alpha 6\beta 4$ increased carcinoma invasion in a PI 3-K-dependent manner and transient expression of a constitutively active PI 3-K increased invasion in the absence of $\alpha 6\beta 4$. Ligation of $\alpha 6\beta 4$ stimulated significantly more PI 3-K activity than ligation of either $\alpha 6\beta 1$ or other $\beta 1$ integrins establishing specificity among integrins for PI 3-K activation. An essential role for PI 3-K in invasion constitutes a novel function for this kinase and implies that downstream effectors of PI 3-K are critical for the invasive process. We provide evidence in fact that the small GTP-binding protein Rac is downstream of PI 3-K in the cells examined and that it is involved in invasion. In contrast, the serine/threonine kinase Akt does not contribute to the invasive process even though it is regulated by PI 3-K and is activated by the $\alpha 6\beta 4$ integrin. Collectively, our findings provide a mechanism for the involvement of $\alpha 6\beta 4$ in promoting carcinoma invasion and invoke a novel function for PI 3-K signaling. These data support the previous findings that the expression of $\alpha 6\beta 4$ is associated with invasive carcinoma. A fascinating problem is raised by these observations because carcinoma progression involves both tumor cell invasion and survival. The recent demonstration that Akt is required for the survival of several cell types (17,18) coupled with our finding that PI-3K is required for invasion suggests that two essential functions of progression may require the products of PI 3-K and that the balance between the use of these pathways may impact tumor cell invasion or survival. These findings are particularly important because they suggest that this $\alpha 6\beta 4$ /PI 3-K signaling pathway is a potential target for inhibiting tumor spread.

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